A PERSISTENT DIURNAL RHYTHM OF CHROMATOPHORIC RESPONSE IN EYESTALKLESS UCA PUGILATOR

H. MARGUERITE WEBB, MIRIAM F. BENNETT AND FRANK A. BROWN, JR.[1]

Department of Physiology and Bacteriology, Goucher College, Towson 4, Md.; Department of Biological Sciences, Northwestern University, Evanston, Ill.; and the Marine Biological Laboratory, Woods Hole, Mass.

On the basis of their response to eyestalk removal crustaceans have been classified into three types (Brown, 1948). The members of Group I, represented by *Palaemonetes,* respond to eyestalk removal by dispersion of the dark pigment and to injection of eyestalk extract by concentration of the pigment. *Crago,* the single member of Group II, responds to eyestalk removal by assuming an intermediate condition with the dark pigment partially dispersed. Group III contains all of the brachyurans except *Sesarma* (Enami, 1951) and is characterized by the complete concentration of the dark pigment on removal of eyestalks. Further investigations have led to the development of a concept of dual hormonal control in members of two of these groups. The evidence supporting such a concept for *Crago* has been reviewed by Brown (1948). In 1952, Brown, Webb and Sandeen demonstrated the presence in the central nervous system of *Palaemonetes* of a red-pigment-dispersing substance and adduced arguments in favor of its normal functioning.

The study of the mechanism of control of the melanophores of *Uca,* a representative of Group III, has been complicated by the presence in these animals of a persistent diurnal rhythm. Under the influence of the rhythmical mechanism the black pigment is dispersed by day and concentrated by night. The extent of dispersion is susceptible to modification by such factors as light, background, and temperature and in at least one species, *Uca pugnax,* an endogenous tidal rhythm has been shown to influence the condition of the chromatophores (Brown, Fingerman, Sandeen and Webb, 1953). All of these factors are thought to act on the chromatophores, at least in part, by virtue of alterations in the blood level of one or more hormones. The eyestalks are known to produce a hormone which causes dispersion of the black pigment. The central nervous system has been shown to contain a substance which disperses the black pigment of eyestalkless animals (Brown, 1948; Sandeen, 1950) but the participation of this substance in physiological color change has not been conclusively demonstrated.

Although all efforts at direct demonstration of a substance acting to concentrate the black pigment of *Uca* have ended in failure, there are cases in which investigators have been led to postulate the existence of such a substance (Brown and Stephens, 1951; Brown and Hines, 1952). Furthermore, Brown and Scudamore (1940) reported observations which suggested that eyestalkless *Uca* do not have their rhythm completely abolished.

[1] This investigation was supported in part by contracts NONR–09703 and NONR–122803 with the Office of Naval Research, and in part by a grant from the Graduate School of Northwestern University.

The data to be reported here contribute to our understanding of two problems: the mechanism of control of the black chromatophores of *Uca pugilator,* and the mechanisms involved in diurnal rhythmicity.

Experimental Procedure

All of these animals used in these experiments were specimens of *Uca pugilator* collected at Chapoquoit beach, near Woods Hole, Mass. during August, 1953. In the laboratory the animals were kept in white enamelled pans with a small amount of water and at a constant illumination of about 2 ft. c.

Two types of experiments were performed. One type involved a study of the changes occurring in chromatophores of legs which had been autotomized and were then maintained in sea water for a period of one hour. The other type of experiment consisted of injection of various concentrations of eyestalk extracts into eyestalkless animals.

Study of changes in chromatophores of isolated legs. Animals were forced to autotomize two or three legs each by applying pressure or by slightly injuring a distal segment of a walking leg. The legs so obtained were placed in sea water and observed at the time of isolation and again after thirty and sixty minutes. The total number of legs removed at any one time varied from six to ten and the legs were taken from two, three or five animals, depending upon the particular experiment. On each occasion a minimum of six legs from normal animals and the same number from eyestalkless animals were observed. Legs were isolated from two such groups at 66 different times; the total number of animals used was 226. The experiments were performed on four different days. In one series legs were removed every hour from 8 P.M. of one day until 8 P.M. of the succeeding day; in the other three series legs were isolated from both normal and eyestalkless animals as follows: 1) hourly from 8 A.M. till the next 1 A.M.; 2) hourly from 7 A.M. until the next 1 A.M.; 3) at 1, 2, 3, and 4 P.M. and at 8 and 9 P.M. of the same day. The eyestalkless donors had been operated on not more than 48 hours and not less than 8 hours before being used in an experiment.

Injection of eyestalk extract. A stock solution was made by grinding 10 dried eyestalks and extracting in one cc. of sea water. This solution was boiled for one minute and then cooled to room temperature. Five-hundredths cc. of this extract (the amount used for a single injection) contained ½ of an eyestalk or ¼ of the normal complement of eyestalk tissue of one animal. Such an extract is said to have a concentration of one quarter. This stock extract was then used to make up a series of concentrations as follows: 1/16, 1/64, 1/128, 1/512, 1/1024, and 1/2048. Seven groups of five eyestalkless animals were injected for each experiment. Each of the five animals in a group received 0.05 cc. of one dilution injected at the base of a walking leg. A control group of five animals received 0.05 cc. of sea water. The state of the chromatophores of each animal was determined at 15 minutes after injection, again at 30 minutes and at 30-minute intervals until they had returned to stage 1.

This experiment was performed four times, using two stock extracts. On one occasion a stock extract was made up in the afternoon, part of it was used immediately and the remainder refrigerated and used in the evening. Again an extract

was made up and part of it used in the evening while a second portion was refrigerated and used the next morning.

Since the chromatophores are initially in stage 1, the observed chromatophore stage minus 1 gives a measure of the dispersion present at any given time of observation. Summing the corrected values obtained during one experiment for any one concentration of extract then gives a measure of the activity of that extract. The activity of each extract tested was calculated in this manner.

Results and Discussion

Figure 1 shows the average stage of the chromatophores of legs isolated from eyestalkless animals, as determined 60 minutes after removal, plotted against time of day at which autotomy occurred. The data used for this curve are those obtained

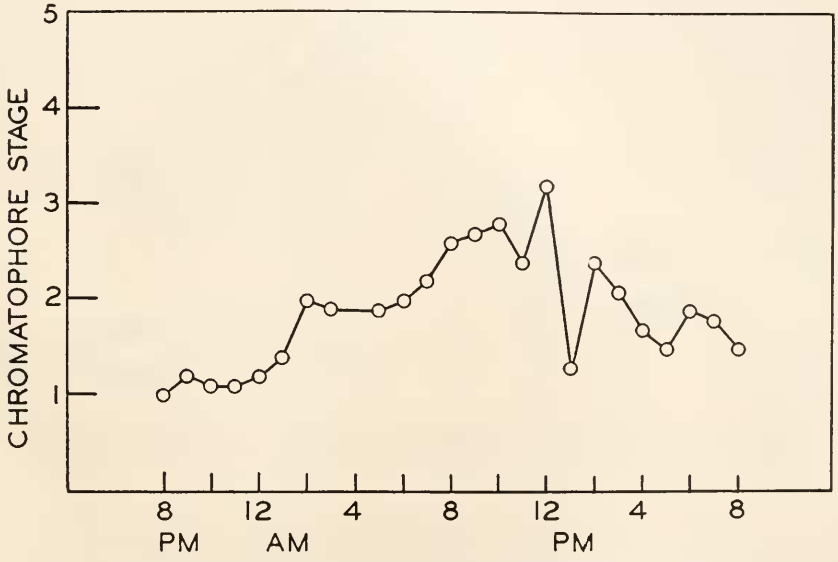

Figure 1. Average index of the melanophores in legs isolated from eyestalkless crabs sixty minutes after isolation at various times during a twenty-four hour period.

in the complete 24-hour series of observations. The other experiments of this type yielded entirely similar results. Since the initial average condition for all chromatophores in legs from eyestalkless animals was stage 1.0 the distance of any point from the abscissa gives a measure of the amount of dispersion occurring at that time of day. It can be seen that during the hours from 8 p.m. to 1 a.m. only very slight dispersion of the pigment occurs in 60 minutes. From 1 a.m. until 12 m. there is a gradual increase in the amount of dispersion observed, while from noon until 8 p.m. a gradually decreasing amount is found.

Curve A of Figure 2 represents the average initial stages of the chromatophores of legs isolated from intact animals plotted against the time of day of removal. Curve B of Figure 2 is obtained by similarly plotting the average stages, at 60

minutes after removal, of the chromatophores of legs from intact animals. These data were obtained on the same day and at the same times as those shown in Figure 1. Results obtained in other experiments of this series were similar to those represented in Figure 2. It can be seen that Curves A and B of Figure 2 are similar in general shape to that describing the conditions for eyestalkless animals. The values are low from 8 P.M. to 2 A.M., increase rather rapidly until about 5 A.M., remain fairly constant until 1 P.M. and then decrease gradually until 8 P.M. The distance between a point of Curve A and the point for the same hour on Curve B gives a measure of the change, concentration or dispersion, occurring in legs removed at that particular hour of the day. It is immediately obvious that the chromatophores in legs isolated from intact animals undergo more or less concentration throughout

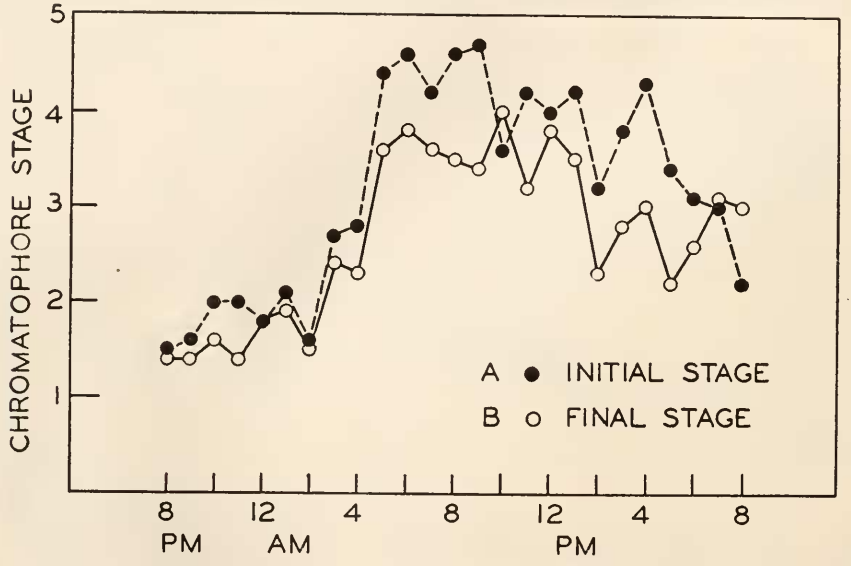

FIGURE 2. Average index of the melanophores in legs isolated from normal crabs at the time of isolation (A) and sixty minutes afterwards (B) as a function of the time of day.

most of the day. In only one case (10 A.M.) is there any noticeable degree of dispersion.

Since all of the isolated legs were kept in sea water and at a constant light intensity, any differences found among experimental groups must be accounted for in terms of differences in the body fluids at the time of isolation. The fact that the chromatophores of legs isolated from eyestalkless animals show different degrees of dispersion at the end of 60 minutes, depending upon time of day of isolation, clearly indicates a diurnal rhythm in eyestalkless animals marked by alterations in the body fluids of the animals.

The results of the injection experiments are presented in Table I. The activity values (calculated by the method previously described) are given for each of the four times the experiment was performed. It is seen that for each of the four lowest

concentrations the activity is lower at night than in the daytime. Although it is possible that some decrease in activity of the extracts occurred between the time of first preparing an extract and the time of injection any such decrease should tend to obscure the differences rather than accentuate them. Thus a decrease with time could result in relatively low daytime values when the extract was made up at night. When the extract was made up in the daytime and used at night the time elapsed was only six hours as compared with 12 hours when the reverse order was followed. The reduction in activity observed is such as would be expected by a two- to four-fold reduction in concentration.

The results obtained in both types of experiments clearly demonstrate that a diurnal rhythm exists in eyestalkless *Uca pugilator*. The changes observed in

TABLE I

Difference between day and night activity for a series of concentrations of eyestalk extract injected into eyestalkless animals

Concentration	Activity of extracts		Difference
	Day	Night	
1/16	18.8	20.0	1.2
	16.0	18.4	2.4
1/64	12.4	13.7	1.3
	12.0	9.0	−3.0
1/128	10.4	8.4	−2.0
	8.0	7.8	−0.2
1/512	6.0	2.2	−3.8
	4.8	3.6	−1.2
1/1024	1.8	0.2	−1.6
	2.6	0.8	−1.8
1/2048	1.6	0.0	−1.6
	1.0	0.0	−1.0

isolated legs show that the rhythm may be characterized as consisting of two distinct phases whose time relationships correspond quite closely with those of the rhythm found under the same conditions for normal animals. From the results obtained following injection of eyestalk extract it is not possible to describe the duration of the phases. The "night injections" were made in both cases shortly after 8 P.M. and it is clear that the response of the eyestalkless assay animals was different from that observed when injections were made in either morning or afternoon.

When an attempt is made to define the nature of the rhythm in terms of substances in the body fluid it is immediately obvious that two substances must be involved. A dispersing substance produced in the eyestalks has long been recognized and it is reasonable to assume that the disappearance of this substance

permits the concentration of pigment that occurs in legs isolated from normal animals. The absence, or presence in smaller concentrations, of dispersing hormone at night might be assumed but whether this is sufficient to account for the observations is questionable. If one postulates a single substance which causes dispersion of the pigment and which is present during the day and absent or reduced in amount at night then one is implicitly assuming that in the absence of any hormone the pigment will be concentrated. Following eyestalk removal the black pigment is maintained in the concentrated condition but disperses in isolated legs. If this concentrated condition is maintained by virtue of the absence of chromatophorotropic hormone then there is no logical explanation for the dispersion that follows isolation. The conclusion is therefore inescapable that the pigment is maintained in the concentrated condition by some factor which is present in the body fluid and which disappears gradually from the isolated legs. The central nervous system is a known source of dispersing hormone but at the present time no source of a black-pigment-concentrating substance has been demonstrated.

Assuming that there are two antagonistic substances which function in the control of the black chromatophores of *Uca pugilator* the rhythm of eyestalkless animals appears to consist of an increased amount of concentrating factor at night. The rhythm of normal animals appears to consist of the production primarily of dispersing hormone in the day phase and primarily of concentrating substance at night. Regardless of the site of production of the concentrating factor it seems likely that control of secretion is nervous and it is clear that the structures of the eyestalk are not essential for continued rhythmicity.

The results obtained on injection of eyestalk extract are consistent with the interpretation that eyestalkless animals possess in their body fluid at night a substance antagonistic to the dispersing hormone of the eyestalk. The fact that no difference was observed with the highest concentrations used may indicate that a maximum response was obtained even at night and that therefore no further response could be expected.

Summary

1. The responses of the black chromatophores of *Uca pugilator* as observed in legs autotomized and maintained in sea water are described.

2. The pigment in legs from normal animals in the day (dispersed) phase becomes concentrated after isolation; that from normal animals in the night (concentrated) phase remains concentrated.

3. The pigment in legs isolated from eyestalkless animals disperses in the daytime and fails to disperse when the legs are removed from 8 P.M. to 2 A.M.

4. The activity on eyestalkless animals of a series of concentrations of eyestalk extract was determined in the daytime and at night. Four of the six concentrations tested were found to be more effective in the daytime than at night.

5. The results clearly demonstrate the existence of a diurnal rhythm in eyestalkless animals and that the structures of the eyestalk are not necessary for this rhythm.

6. The data provide strong evidence that a black-pigment-concentrating substance participates in the regulation of the chromatophore system of these animals.

LITERATURE CITED

Brown, F. A., Jr., 1948. Hormones in crustaceans. Chapter V, The Hormones. Academic Press, Inc. New York.

Brown, F. A., Jr., M. Fingerman, M. I. Sandeen and H. M. Webb, 1953. Persistent diurnal and tidal rhythms of color change in the fiddler crab, *Uca pugnax*. *J. Exp. Zool.*, **123**: 29–60.

Brown, F. A., Jr., and M. N. Hines, 1952. Modifications in the diurnal pigmentary rhythm of *Uca* effected by continuous illumination. *Physiol. Zool.*, **25**: 56–70.

Brown, F. A., Jr., and H. H. Scudamore, 1940. Differentiation of two principles from the crustacean sinus gland. *J. Cell. Comp. Physiol.*, **15**: 103–119.

Brown, F. A., Jr., and G. C. Stephens, 1951. Studies of the daily rhythmicity of the fiddler crab, *Uca*. Modifications by photoperiod. *Biol. Bull.*, **101**: 71–83.

Brown, F. A., Jr., H. M. Webb and M. I. Sandeen, 1952. The action of two hormones regulating the red chromatophores of Palaemonetes. *J. Exp. Zool.*, **120**: 391–421.

Enami, M., 1951. The sources and activities of two chromatophorotropic hormones in crabs of the genus *Sesarma*. I. Experimental analyses. *Biol. Bull.*, **100**: 28–43.

Sandeen, M. I., 1950. Chromatophorotropins in the central nervous system of *Uca pugilator* with special reference to their origins and actions. *Physiol. Zool.*, **23**: 337–352.